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Metabolism and Excretion of Injected [^{3}H]-Ecdysone by Female Lobsters, *Homarus americanus*

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Abstract. The dynamics of ecdysteroid metabolism and excretion were followed in adult lobsters, *Homarus americanus*. Females at five different molt stages were injected with [^{3}H]-ecdysone. Levels of [^{3}H]-20-hydroxyecdysone (20E), converted from [^{3}H]-ecdysone, rose rapidly and remained significantly higher in premolt stages D_0 and D_1. In contrast, significant increases in the levels of highly polar ecdysteroid metabolites (HP) occurred primarily in stages A and C. Changes in the hemolymph levels of 20E and HP in hemolymph over the molt cycle suggest additional metabolic mechanisms by which the titers of active molting hormones can be regulated.

Excretion of [^{3}H]-ecdysteroids was slower during early premolt stages D_0 and D_1, suggesting that this reduced rate may be an additional mechanism for regulating ecdysteroid titers. Study of [^{3}H]-ecdysteroids indicated that metabolism proceeds primarily to HP that are excreted in the urine with unaltered ecdysteroids. An additional ecdysteroid metabolic route was found in the midgut gland; this route removes ecdysteroids from the hemolymph and transforms them into apolar metabolites prior to their excretion in the feces. This route is similar to that previously found for ingested [^{3}H]-ecdysone, which was converted to apolar conjugates without further absorption.

Introduction

The first ecdysteroid isolated from a decapod crustacean was 20-hydroxyecdysone (20E) by Hampshire and Horn (1966). Since that report, nearly 20 different ecdysteroids have been identified from over 25 crustacean species (see Chang, 1989, for review). 20E has been reported to be the putative active molting hormone, because it specifically alters premolt changes in kinase activities and protein synthesis in epidermal tissues (Christ and Sedlmeier, 1987; Traub *et al.*, 1987).

The primary ecdysteroid product of the molting gland, or Y-organ, is thought to be ecdysone (Chang and O'Connor, 1977). Additional evidence suggests that the Y-organ secretes other ecdysteroids, namely 25-deoxyecdysone (Lachaise *et al.*, 1989) and 3-dehydroecdysone (Spaziani *et al.*, 1989). A single hydroxylation step converts ecdysone and 25-deoxyecdysone to the more active products 20E and ponasterone A (P), respectively. Further metabolism proceeds by additional hydroxylation steps, formation of acids, and conjugation to form polar and apolar products (McCarthy, 1980, 1982; Lachaise and Lafont, 1984; Connat and Diehl, 1986; Snyder and Chang, 1991a, b).

Many decapod tissues absorb [^{3}H]-ecdysone or [^{3}H]-20E from the hemolymph (Kuppert *et al.*, 1978; McCarthy, 1980, 1982) and metabolize these injected ecdysteroids *in vitro* (Lachaise and Lafont, 1984). The metabolism of ecdysteroids has been studied in greater detail in insects, and the structural identities of many metabolites have been confirmed by mass spectrometry, nuclear magnetic resonance, and other chemical techniques (reviewed by Koolman and Karlson, 1985).

A characteristic pattern of hemolymph ecdysteroid titers defines the crustacean molt cycle; *i.e.*, titers are low until the final large premolt peak, and this peak is followed by a rapid decline just prior to ecdysis (Chang, 1989). Recently, the decapods *Uca pugilator* and *Homarus americanus* were reported as having other significant titer variations during their molt cycles (Hopkins, 1986; Snyder and Chang, 1991a). Hemolymph 20E levels in the lobster

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Abbreviations: 20,26E, 20,26-dihydroxyecdysone; 20E, 20-hydroxyecdysone; 20EA, 20-hydroxyecdysonoic acid; HP, highly polar ecdysteroid metabolites; HPLC, high-performance liquid chromatography; P, ponasterone A; RP, reverse phase; T, triol (22,25-dideoxyecdysone).

H. americanus drop precipitously in late premolt, and the drop is associated with an increase in the titer of highly polar metabolites (Snyder and Chang, 1991a). The titers of hemolymph ecdysteroids decrease both in late premolt and when regenerating limb buds autotomize. These changes are explicable by increases in both the metabolism of ecdysteroids to polar conjugates and the excretion of ecdysteroids (McCarthy, 1980, 1982). Other than control of the Y-organ by molt-inhibiting hormone, additional controlling mechanisms for the regulation of ecdysteroid titers are little known in crustaceans (Chang, 1989).

Excretion pathways for ecdysteroids in crustaceans have received little attention. When decapods were injected with [^{3}H]-ecdysone, [^{3}H]-20E, or [^{3}H]-P, much of the radiolabel appeared in the surrounding water within 1 to 48 h (Lachaise *et al.*, 1976; Kuppert *et al.*, 1978; Buchholz, 1982; Lachaise and Lafont, 1984). We recently cannulated both the antennal gland and anus and found that ecdysteroids are excreted both in urine and feces, although urine is the major route (Snyder and Chang, 1991b). The gut also excretes ecdysteroids from the hemolymph, in addition to playing an important role in detoxifying (by apolar conjugation) and excreting ingested ecdysteroids (Snyder and Chang, 1991b). That the gut metabolizes ecdysteroids, whether ingested, endogenous, or injected, has been found in several arthropods (Isaac and Slinger, 1989).

We have injected ecdysteroids into lobsters at five stages in the molt cycle, and have determined the levels of metabolites produced. These studies have revealed the changes in ecdysteroid metabolism that occur during the molt cycle. In addition, we have cannulated the anus and urinary pores, collected the radiolabeled metabolites, and thus elucidated the excretory routes for injected ecdysteroids.

Materials and Methods

Animals

Adult female *Homarus americanus* (420–570 g wet wt.) were either obtained from a seafood supplier (Net Result, Martha's Vineyard, Massachusetts) or were reared at the Bodega Marine Laboratory (Chang and Conklin, 1983; Conklin and Chang, 1983). No differences were observed between the lobsters obtained from these two sources. Only non-reproductive lobsters were used in this study to avoid ovarian influences on ecdysteroid dynamics (Lachaise *et al.*, 1981). They were maintained in a flow-through system at 12 ± 3.5°C on a 16L:8D photoperiod and fed a mixed diet of frozen fish, shrimp, and live mussels thrice weekly. The lobster premolt stages $D_0^{1.0}$, D_1', D_1'', D_1''', D_2', and D_2'' of Aiken (1973) are reported here as stages D_0^1, D_1^1, D_1^2, D_1^3, D_2^1, and D_2^2, respectively. Staging of postmolt and early intermolt was made according to the degree of softness of the carapace and chelae as reported by Stevenson (1968).

Cannulation was accomplished as follows. A lobster was restrained on its dorsum on a bed of ice. Both antennal gland pores were then externally cannulated by the methods of Holliday (1977). In addition, a cannula was inserted into the anus and held in place with cyanomethacrylate glue. The cannula (3.18 × 4.76 × 80 mm, i.d. × o.d. × l) was open-ended and initially filled with air. When properly attached, water did not enter the cannula. When the lobster defecated, the feces expelled air distally from the cannula. The remaining air in the cannula protected the feces from being contaminated by seawater. Feces were collected from the cannula after it was removed from the animal.

Injections

[23,24-^{3}H]-ecdysone (89 Ci/mmol, New England Nuclear) was purified by high-performance liquid chromatography (HPLC), dissolved in lobster saline (Mykles, 1980), and 3–4 μCi injected into the hemocoel at the base of the fourth pereiopod. The injected ecdysone did not raise the levels of circulating ecdysteroids above those previously observed (Snyder and Chang, 1991a). Injected animals were in stages A–B, C_4, D_0^1, D_1^1, and D_2^2–D_3^1. The hemolymph was sampled at 1, 4, 12, 24, 48, 72, and 96 h after injection, and all excreta were collected daily. Each of the collected samples was extracted in methanol and prepared for HPLC and liquid scintillation spectrometry, as described previously (Snyder and Chang, 1991a,b).

In one experiment, juvenile female lobsters (stage C_4, 29–46 g wet wt.) were injected with 1 μCi of [^{3}H]-ecdysone. At 1 h and 10 days, four lobsters were sacrificed, and the midgut glands, ovaries, abdominal muscles, hindguts, antennal glands, epidermal tissues of the cephalothorax, and the remaining carcasses were extracted in 100% methanol. Following two re-extractions and centrifugations (10 min, 4100 × *g*), portions of the resultant supernatants were subjected to liquid scintillation spectrometry for the determination of total radioactivity per tissue. Because the sample extracts were highly diluted, no variations in counting efficiency were observed. As a positive control, we added [^{3}H]-ecdysone to non-radiolabeled tissues prior to the extraction steps and thus determined that our tissue extraction efficiencies were 80–90%.

HPLC

Samples of individual hemolymph, urine, and fecal extracts were dissolved in the appropriate solvent, centrifuged, and the supernatant injected directly onto a Waters C_{18} μBondapak column (3.9 mm I.D. × 30 cm). One of the following reverse phase elution conditions was used:

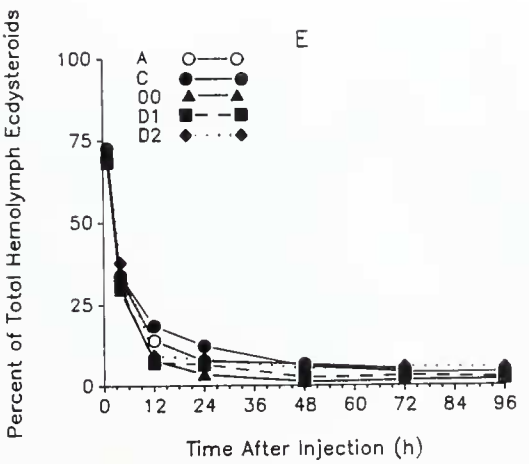

Figure 1. Changes in the hemolymph level of injected [^{3}H]-ecdysone (E) as a function of time and molt stage. [^{3}H]-ecdysone was injected at time zero. Concentration of labeled ecdysone in the hemolymph of lobsters is expressed as a percentage of the total [^{3}H]-ecdysteroids, and determined by methanolic extraction of hemolymph samples followed by scintillation counting of reverse phase-HPLC fractions. Samples were separated with gradient systems #1 or #2 (see text) with either (or both, for some samples) methanol or acetonitrile as the solvent. Molt stages A, C, D_0, D_1, and D_2 refer to the morphological designations of Aiken (1973). Sample sizes were as given for Table I with the addition of n = 3 for stage A. Standard deviation bars are omitted for clarity.

(1) a 35 min linear gradient of 20–100% methanol in water at 1.0 ml/min (1.0 min fractions); (2) a linear gradient of 20–100% acetonitrile in 20 m*M* Tris, pH 7.5, at 1.0 ml/min (1.0 min fractions collected); or (3) a linear gradient of 20–100% methanol in 20 m*M* Tris, pH 7.5, at 1.0 ml/min (1.0 min fractions collected). In all cases, we employed a Waters HPLC system. Duplicate samples from each fraction were analyzed by scintillation spectrometry. The sum of the radioactive ecdysteroids recovered in the HPLC-fractions was equal to 70–85% of the total, unfractionated radioactivity. The amount of each [^{3}H]-ecdysteroid metabolite was expressed as the percentage of the total radioactivity, and the values at each time point were compared statistically by ANOVA and Scheffé tests of arcsine transformed values (Sokal and Rohlf, 1969).

Enzymatic hydrolysis

The fractions resulting from HPLC that contained ecdysteroids of greater polarity than 20E are designated "polar fractions." The polar fractions from individual samples of urine and feces were pooled and then incubated, at 37°C for 24 h, in 1.0 ml sodium acetate buffer (50 m*M*, pH 5.5) containing 3.0 mg/ml type H-2 *Helix pomatia* sulfatase (Sigma). Apolar fractions from fecal samples were dissolved in ethanol (5% v/v in final hydrolysis mixture) with or without addition of enzymes, and incubated for 72 h (Whiting and Dinan, 1988). These modifications increased the hydrolysis of apolar material (Whiting and Dinan, 1988; Snyder and Chang, 1991b). After the addition of three volumes of methanol to terminate the reactions, the samples were centrifuged at 4100 × *g*, re-extracted twice, and the pooled supernatants evaporated under reduced pressure and analyzed by HPLC-scintillation spectrometry.

Results

Hemolymph ecdysteroids

Changes in hemolymph ecdysteroid metabolites were followed for 96 h after the injection of [^{3}H]-ecdysone. Figure 1 shows the rate of disappearance of ecdysone from the hemolymph. The loss of ecdysone, as a percentage of the total hemolymph [^{3}H]-ecdysteroids, was not significantly different from one molt stage to another. Within 1 h, ecdysone levels had fallen to about 70% of the total. Levels dropped dramatically to 6.5–12.2% by 24 h. Levels of [^{3}H]-ecdysone did not fall to zero and were still 2.5–5.5% of the total at 96 h.

Hemolymph [^{3}H]-20E levels were also followed after the injection of [^{3}H]-ecdysone (Fig. 2). By 1 h, [^{3}H]-20E percentages were 17–27% of the total [^{3}H]-ecdysteroids and not significantly different among the different molt stages. At 4 and 12 h, lobsters in stages D_0 and D_1 had higher percentages of labeled 20E (relative to other ecdysteroids) than at other molt stages. Levels of 20E for both stages (≥75% of the total) were consistently higher

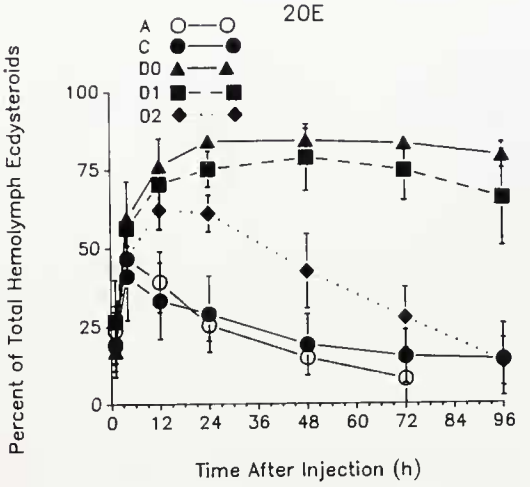

Figure 2. Change in the hemolymph level of radiolabeled 20-hydroxyecdysone (20E) as a function of time and molt stage. [^{3}H]-ecdysone was injected at time zero. Concentration of labeled 20E in the hemolymph of lobsters is expressed as a percentage of the total [^{3}H]-ecdysteroids. Separation and quantification conditions are as listed in Figure 1 and Materials and Methods. Sample sizes are as given for Table I with the addition of n = 3 for stage A. Bars indicate one standard deviation from the mean.

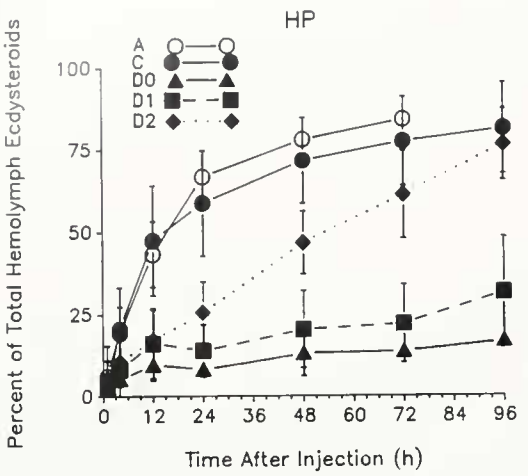

Figure 3. Change in the hemolymph level of radiolabeled highly polar ecdysteroid metabolites (HP) as a function of time and molt stage. [^{3}H]-ecdysone was injected into lobsters at time zero. Concentration of labeled HP in the hemolymph is expressed as a percentage of the total [^{3}H]-ecdysteroids. Separation and quantification conditions are as listed in Figure 1 and Materials and Methods. Sample sizes are as given for Table I with the addition of n = 3 for stage A. Bars indicate one standard deviation from the mean.

than those of other stages through 96 h. Premolt lobsters in stages D_1 and D_2 had similar 20E percentages at least until 12 h post-injection of [^{3}H]-ecdysone. By 24 h, the [^{3}H]-20E in the D_2 lobsters started to drop dramatically, reaching, by 72–96 h, levels equivalent to those in stages A and C. Levels of [^{3}H]-20E were significantly lower in D_2 than either D_0 or D_1 lobsters by 24 h. The concentrations of [^{3}H]-20E in stages A and C changed together throughout the 96 h experiment. Levels for those two stages peaked (41–47%) at 4 h, and then dropped to 7.9–11.1% of the total [^{3}H]-ecdysteroids by 72 h. Stage A and C [^{3}H]-20E levels were significantly lower than those of all other stages until 96 h post-injection, when stage D_2 lobsters had similar values. These data indicate that the rate of 20E loss becomes significantly faster as lobsters approach stage D_3.

Changes with time in the percentages of highly polar ecdysteroid metabolites (HP) were the converse of those in 20E (Fig. 3). All molt stages were similar from 1–4 h post-injection of [^{3}H]-ecdysone. The hemolymph percentages of [^{3}H]-HP in stages D_0 and D_1 lobsters barely increased. This is because [^{3}H]-20E percentages remain high in these two stages through 96 h (Fig. 2). The percentage of [^{3}H]-HP in late premolt stage D_2 lobsters was equivalent to those in D_0 and D_1 animals through 12 h (8–17% of the total), then rose to a significantly higher level by 48 h. At 72 h, stages D_2, A, and C had similar [^{3}H]-HP percentages. Stage A and C lobsters both showed rapid increases in [^{3}H]-HP percentages that were significantly higher (increasing to >75% of the total) than those in other molt stages.

Ecdysteroid excretion

Table I gives the data for total excretion of [^{3}H]-ecdysteroids, as a percentage of the injected dose, during the first 6 or the first 15 days after injection of [^{3}H]-ecdysone. Lobsters in stages D_0 and D_1 excreted significantly less ecdysteroids (12%) in the first 6 days after injection than those in stages C or D_2 (30%). By 15 days, D_0 lobsters had excreted significantly less [^{3}H]-ecdysteroids (28%) than those in stage D_1 (39%) and C (45%). Urine was always the major route for ecdysteroid excretion (Table I). In stages C and D_2, 90–96% of the radiolabel was excreted in the urine. Significantly more [^{3}H]-ecdysteroids were excreted in the feces of stage D_0 and D_1 lobsters than at stages C or D_2.

Ecdysteroid metabolites

Lobsters in stages C through D_1 were injected with [^{3}H]-ecdysone. Ninety-six hours later, the profiles of ecdysteroid metabolites in hemolymph, urine, and feces were examined. The data for injections into stages C (Fig. 4) and D_1 (Fig. 5) were similar to those for injections into stages D_2 and D_0, respectively. Therefore, profiles for the latter injections are not shown. At least four HP were resolved by RP-HPLC. Besides 20E, ecdysone, and P, two of these HP were tentatively identified as epimers of 20-hydroxyecdysonoic acid (20EA) and 20,26-dihydroxyecdysone (20,26E). The characterizations were based on co-elution with authentic standards in at least two different solvent systems; normal phase separations were also performed on some samples (data not shown). In addition, two other ecdysteroid metabolites were designated as "highly polar

Table I

Percentage of radioactivity recovered from excreta of adult lobsters following injection of [^{3}H]-ecdysone

		Urine plus feces[2]		Days 1–15[3]	
Molt stage[1]	n	Days 1–6	Days 1–15	Urine	Feces
C	5	30.0 ± 4.1[a]	45.2 ± 3.7[a]	90.2 ± 2.9[a]	9.8 ± 2.9[a]
D_0	3	11.5 ± 2.8[b]	28.2 ± 4.7[b]	80.6 ± 0.8[b]	19.4 ± 0.8[b]
D_1	4	12.0 ± 2.1[b]	38.7 ± 5.0[a]	73.3 ± 3.5[c]	26.7 ± 3.5[c]
D_2	4	30.6 ± 3.9[a]	—	95.5 ± 2.1[d]	4.5 ± 2.1[d]

[1] Molt stage designations are those of Aiken (1973). Postmolt stages A and B were not monitored for [^{3}H] excretion. Values with different superscript letters (within a column) indicate significant differences ($P < 0.05$).

[2] Sum of the radioactivity excreted in both the urine and feces in either the first 6 or the first 15 days following [^{3}H]-ecdysone injection. Late premolt stage D_2 lobsters were followed only for the first 6 days (after which they molted).

[3] Percentages of total radioactivity recovered in either the urine or feces over the entire experiment (days 1–15).

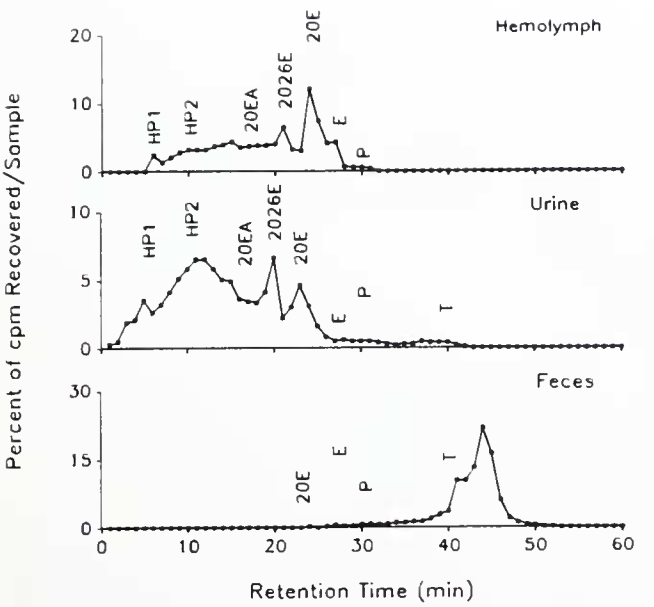

Figure 4. Reverse phase-HPLC-scintillation spectrometric analyses of hemolymph, urine, and fecal [^{3}H]-ecdysteroids. Samples were obtained from a stage C_4 lobster 96 h after injection of [^{3}H]-ecdysone. Separation conditions are described in Figure 1. The retention times of authentic 20-hydroxyecdysonoic acid epimers (20EA), 20,26-dihydroxyecdysone (20,26E), 20-hydroxyecdysone (20E), ecdysone (E), ponasterone A (P), and 22,25-dideoxyecdysone (triol, T) are shown. Two highly polar ecdysteroid products, which include conjugates and non-enzyme-hydrolyzable metabolites, are labeled as HP1 and HP2.

1" (HP1) and "highly polar 2" (HP2). The most polar metabolite, HP1, was a mixture of conjugates of 20EA, 20,26E, and 20E and also contained *Helix pomatia* enzyme-resistant compounds. The urine of stage D_2 lobsters contained significantly more HP1 material as enzyme-hydrolyzable conjugates than any other molt stage. This result indicates that ecdysteroid conjugation may increase in the late premolt stage. Hydrolysis of HP2 also yielded 20EA, 20,26E, 20E, and non-hydrolyzable material. Raising both the concentration of enzymes and incubation times failed to increase the hydrolysis of HP1 and HP2, supporting the idea that they may also contain other types of ecdysteroid metabolites. There were no significant differences between any of the molt stages in 20E and ecdysone excretion as percentage of the total ecdysteroids excreted per day in urine. But, since the excretion rates in stages C and D_2 were significantly higher in days 1–6 (Table II) than those in D_0 and D_1, more free 20E and ecdysone were excreted in the urine of the former stages.

The hemolymph of stage C lobsters, 96 h after injection, contained mainly HP2, 20EA, 20,26E, and 20E. Smaller quantities of HP1, ecdysone, and P were also present. The urine of stage C animals contained almost 90% HP with HP2, 20,26E, and 20E in similar proportions. The feces contained >95% apolar material; one component eluted with a retention time similar to that of 22,25-dideoxyecdysone (triol, T). Only small percentages of [^{3}H]-20E, ecdysone, and P were ever found in the feces of any molt stage.

Metabolite profiles for stage D_2–D_3 are shown in Figure 5. The major hemolymph ecdysteroid at this stage was 20E, with smaller amounts of HP1, HP2, 20,26E, and ecdysone. The urinary profile is also shown. Fecal [^{3}H]-ecdysteroids were >99% apolar products. Hydrolysis of the fecal apolar material yielded a number of products (Fig. 6). The profiles of fecal ecdysteroid conjugates varied according to molt stage. Figure 6 shows the profile of a premolt stage D_1 lobster fecal sample 10 days post-injection; at this time, 20E was the major hemolymph metabolite. Hydrolysis yielded a large percentage of 20E, and smaller amounts of HP1, ecdysone, P, T, and unhydrolyzed apolar components. During intermolt stage C, hydrolysis of fecal apolar ecdysteroids resulted in a higher percentage of free HP (data not shown).

The uptake and metabolism of [^{3}H]-ecdysone injected into hemolymph was studied in juvenile lobsters in intermolt stage C (Table II). By 1 h after injection, only 33% of the radiolabel remained in the hemolymph. Tissues such as hindgut, antennal glands, immature ovaries, and epidermis all contained <1% of the injected dose at 1 h. Only abdominal muscle (3.7%), midgut gland (4.5%), and

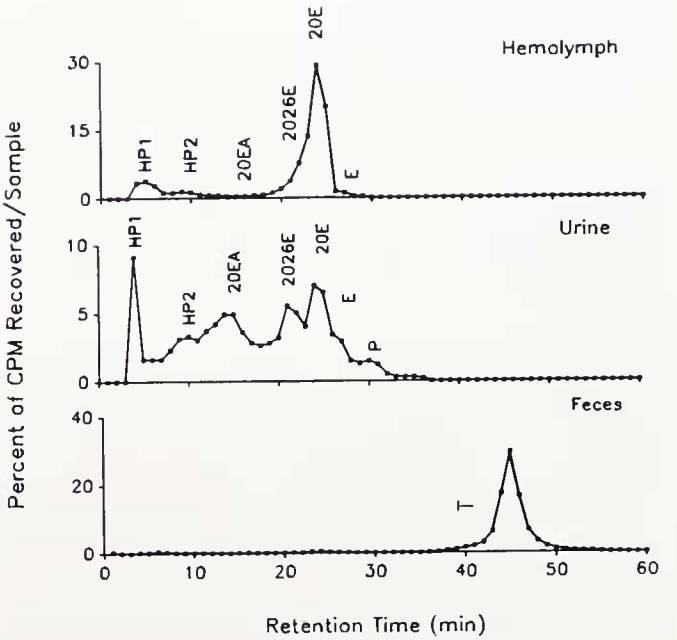

Figure 5. Reverse phase-HPLC-scintillation spectrometric analyses of hemolymph, urine, and feces from a lobster at stage D_2–D_3 (when the hemolymph ecdysteroids reach a maximum). The animal was injected with [^{3}H]-ecdysone in stage D_1 and sampled 96 h later. Separation conditions are described in Figure 1. Abbreviations for the various ecdysteroids are as in Figure 4.

carcass (45%) contained appreciable radioactivity at 1 h. When calculated as percentage of dose per gram wet weight of tissue, the largest amounts were found (after 1 h) in the antennal glands (16%) and ovaries (11%). The smallest quantities on a per weight basis were found (after 1 h) in muscle (1.5%) and carcass (1.8%).

By 10 days post-injection, about 41% of the injected dose had been lost due to excretion. Concomitantly, most tissues had very low levels of radioactivity; hindgut, antennal glands, epidermis, ovaries, and muscle all contained 0.1–0.2% of the initial label. Higher levels were found in hemolymph (1.0%), midgut gland (5.2%), and carcass (14%). On a wet weight basis, significantly higher levels were associated with hindgut, antennal glands, and midgut gland (30–32%). Clearly, by 10 days, the midgut gland had concentrated much more [^{3}H]-ecdysteroids than any other single tissue examined.

At 1 h and 10 days, 11% and 38% of the injected dose, respectively, could not be accounted for by either losses in methanol extraction (81% efficiency), leakage from the injection site (about 2.5%, as judged from counts of absorbent paper held on the wound for 30 s after injection, and from counts of the seawater bath 1 h later), or from adherence to the injection needle (about 5%). Possible explanations for these unexplained losses are [^{3}H] exchange or the activity of side-chain cleaving enzymes; the latter has been suggested to occur in decapod crustaceans (Lachaise and Lafont, 1984) and in other arthropods (Koolman and Karlson, 1985).

Some tissue extracts from 1 h and 10 days after injection of juvenile lobsters were also studied by RP-HPLC (Fig. 7). In 1 h, the largest amount of [^{3}H]-ecdysteroids in hemolymph remained as ecdysone, followed by smaller quantities of 20E and P. Only about 2% of the dose was found as HP in hemolymph 1 h after injection. Abdominal muscle contained higher levels of 20E than hemolymph, but ecdysone was still the major ecdysteroid at 1 h. At 1 h, muscle contained slightly higher (about 5%) levels of HP than did hemolymph. Large amounts of 20,26E, and

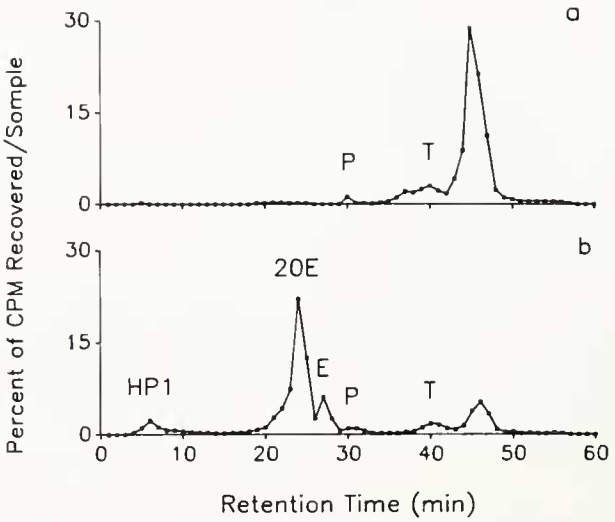

Figure 6. Enzyme treatment of fecal [^{3}H]-ecdysteroids from a stage D_1 lobster. Ecdysteroids were isolated from feces and incubated without (a) or with (b) *Helix pomatia* sulfatase. The samples were then analyzed by reverse phase-HPLC. Separation conditions are described in Figure 1. Abbreviations for the various ecdysteroids are as in Figure 4.

Table II

Recovery of radioactivity in tissues of juvenile lobsters injected with [^{3}H]-ecdysone[1]

Sample	n	H	HG	AG	EP	OV	M	MG	C
1 h[2]	4								
% of injected dose		33.1 ± 3.0^e	0.2 ± 0^a	0.4 ± 0.1^b	0.9 ± 0.1^c	0.9 ± 0.2^c	3.7 ± 0.8^d	4.5 ± 0.6^d	45.4 ± 6.1^f
%/g wet wt.		3.3 ± 1.0^{a,c}	3.5 ± 0.8^{a,c}	15.6 ± 6.1^b	3.8 ± 1.0^a	10.8 ± 2.1^b	1.5 ± 0.8^{c,d}	2.8 ± 0.9^{a,c,d}	1.8 ± 0.2^d
10 days[2]	4								
% of injected dose		1.0 ± 0.2^c	0.2 ± 0.1^a	0.1 ± 0^a	0.1 ± 0^a	0.1 ± 0^a	0.4 ± 0^b	5.2 ± 1.1^d	13.7 ± 2.4^e
%/10 g wet wt.		1.1 ± 0.2^a	30.3 ± 25.4^d	32.4 ± 1.7^d	4.0 ± 1.3^b	11.7 ± 7.8^b	5.2 ± 9.1^{a,b,c}	32.3 ± 5.1^d	6.0 ± 0.4^c

[1] Lobsters were injected with [^{3}H]-ecdysone, sacrificed at either 1 h or 10 days; methanolic extracts were then made of the various tissues: (H = hemolymph, HG = hindgut, AG = antennal glands, EP = epidermis of cephalothorax, OV = ovaries, M = abdominal muscle, MG = midgut gland, and C = remaining carcass). The radioactivity recovered from extracts was computed from determinations on aliquots and expressed as a percentage of the total injected dose.

[2] The data are presented, either as a percentage of the injected dose, or as a percentage of the dose per gram (for 1 h), or per 10 g (for 10 days) of wet tissue. Values are means (±1 standard deviation). Different superscript letters denote values that are significantly different ($P < 0.05$) from each other (within a row); analysis by ANOVA followed by Scheffé tests of the arcsine transformations of the percentage values (Sokal and Rohlf, 1969).

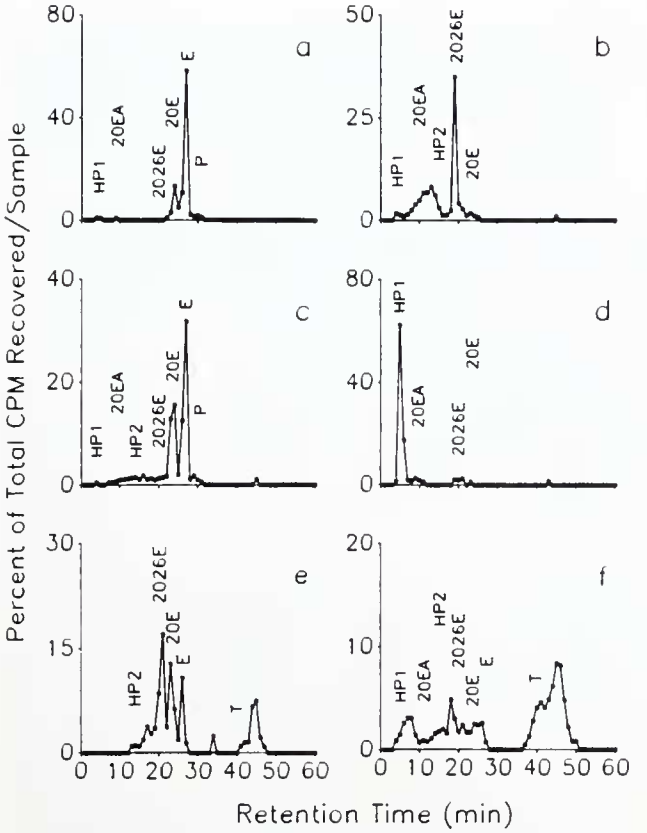

Figure 7. Reverse phase-HPLC chromatograms of extracts of tissues from juvenile stage C_4 lobsters. Hemolymph (a,b), abdominal muscle (c,d), and midgut gland (e,f) were taken at either 1 h (a,c,e) or 10 days (b,d,f) after injection with [^{3}H]-ecdysone. Samples were separated using gradient #3. Abbreviations for the various ecdysteroids are as in Figure 4.

approximately equivalent amounts of 20E, ecdysone, and apolar products were present in midgut gland at 1 h after injection. Smaller amounts of HP2, and a peak eluting between P and T, were also found in the midgut gland at 1 h.

By 10 days, hemolymph contained mostly HP with a large peak of 20,26E and smaller quantities of HP1, 20EA, HP2, and 20E. In contrast, a large peak of HP1 and very small amounts of 20EA, 20,26E, and 20E were present in the muscle at 10 days. A large amount of apolar material and smaller quantities of HP1, 20EA, 20,26E, 20E, and ecdysone were found in the midgut gland at 10 days.

Discussion

At all molt stages, ecdysone was very rapidly eliminated from lobster hemolymph. The rapid loss of [^{3}H]-ecdysone was not unexpected, because ecdysone titers in *H. americanus* (as determined by RIA) were never more than 19% of the total ecdysteroids in hemolymph (Snyder and Chang, 1991a). Ecdysone also has a short half-life in the crab *Gecarcinus lateralis* (McCarthy, 1980, 1982) and in several insect species (reviewed by Koolman and Karlson, 1985; Koolman and Walter, 1985). The activity of ecdysone 20-monooxygenase, which converts ecdysone to 20-hydroxyecdysone (20E), probably has a role in changing the levels of ecdysone in lobsters, as it does in several insects (Smith *et al.*, 1983; Mitchell and Smith, 1988). Young (1976) treated blowflies with supraphysiological doses of ecdysone, and demonstrated that alterations in the conversion rates of ecdysone to 20E could not be explained by saturation of metabolizing sites. Lachaise *et al.* (1976) reported that the rate of conversion of ecdysone to 20E was slower in postmolt and intermolt than in premolt stage crabs. Soumoff and Skinner (1988) demonstrated that enzyme activity varied with molt cycle in *G. lateralis* and that the variations were lowest in late premolt and postmolt. Additionally, Chang and O'Connor (1978) showed that 20-hydroxylation activity increased by four times in the testes of crabs (*Pachygrapsus crassipes*) that had undergone eyestalk ablation. The variation in ecdysone 20-monooxygenase during the crustacean molt cycle is still not understood.

Coincident with the drop in [^{3}H]-ecdysone were increases in [^{3}H]-20E. The primary metabolite of ecdysone in other crustaceans has been shown to be 20E (Lachaise *et al.*, 1976; Chang and O'Connor, 1978; Kuppert *et al.*, 1978; McCarthy, 1980, 1982; Buchholz, 1982). The relative amounts of [^{3}H]-20E, following the peak of conversion from [^{3}H]-ecdysone at 4–12 h, were significantly higher in the premolt stages of lobsters. In the late premolt stage D_2, there was a rapid loss of [^{3}H]-20E after 24 h similar to that exhibited in stages A and C after 4 h. These results are suggestive of a mechanism that regulates ecdysteroid metabolism around the time of the late premolt peak in the hemolymph (Snyder and Chang, 1991a). McCarthy (1982) reported long hemolymph 20E half-lives for *G. lateralis* in early-mid premolt. Long half-lives for 20E in early premolt crabs were significantly reduced by the autotomy of partially regenerated limbs, suggesting that other controls of ecdysteroid metabolism exist (McCarthy, 1980). Others have reported that 20E catabolism in insects can vary over larval-pupal stages (reviewed by Lehmann and Koolman, 1989). As in the lobster molt cycle, 20E was lost at a faster rate when molting hormone titers were increasing in the blowfly *Calliphora vicina* (Young, 1976; Young and Young, 1976; Koolman and Walter, 1985) and in the tobacco hornworm *Manduca sexta* (reviewed by Gilbert, 1989). The potential roles of 20E catabolic activity in the regulation of crustacean ecdysteroid titers require further study.

Levels of highly polar [^{3}H]-ecdysteroid metabolites (HP), such as 20-hydroxyecdysonoic acid (20EA), 20,26-dihydroxyecdysone (20,26E), and conjugates, increased

in hemolymph after increases in 20E. In *H. americanus*, hemolymph metabolites appeared in the following order: ecdysone → 20E → HP. The metabolism of a single injected dose of [^{3}H]-ecdysone appeared to mimic normal ecdysteroid metabolism in lobsters. Highly polar metabolites were the major circulating ecdysteroids in all lobster molt stages, except during mid-late premolt, the period when the major peak of ecdysteroids occurs in the hemolymph (Snyder and Chang, 1991a). However, of the metabolites detected by RIA in lobster hemolymph, urine, and feces (Snyder and Chang, 1991a,b), not all were found after the injection of [^{3}H]-ecdysone. A few unidentified metabolites eluting from the RP column between ecdysone and P were absent in the present study. Gilbert (1989) advised caution in the interpretation of [^{3}H]-ecdysone injection experiments, as similar incomplete ecdysteroid profiles were found in *Manduca sexta*.

Injections of [^{3}H]-ecdysone into cannulated lobsters confirmed that the likely route for excretion of ecdysteroid metabolites (HP, and unconjugated 20E, ecdysone, and P) from hemolymph is via the urine in all molt stages. Equivalent results were found when excreta were assayed by RIA throughout the molt cycle (Snyder and Chang, 1991b). Others have shown that HP were formed by decapod crustaceans following the injection of [^{3}H]-ecdysone or [^{3}H]-P (Lachaise *et al.*, 1976; Kuppert *et al.*, 1978; McCarthy, 1980, 1982; Buchholz, 1982; Lachaise and Lafont, 1984). Some of these metabolites, including conjugates, have also been found in the seawater surrounding the animals (Buchholz, 1982; Lachaise and Lafont, 1984). The only differences related to the molt cycle that were discovered in lobster urine were in the amounts of HP conjugates, which were much higher in late premolt (stage D_2) lobsters. It may be that highly polar conjugates are destined for excretion only in non-reproductive lobsters, and that excretion is more significant during the rapid decline in ecdysteroid titer just prior to ecdysis. Lachaise and Lafont (1984) found similar increases in highly polar ecdysteroid conjugates in late premolt crabs (*Carcinus maenas*) after injection of ponasterone A. Polar conjugates are loaded into vitellogenic ovaries, and thus may be potential sources of ecdysteroids for developing crustacean embryos (Lachaise *et al.*, 1981; Spindler *et al.*, 1987).

The data on excretion rates (Table I), suggest that lobsters have an additional mechanism for regulating ecdysteroid levels. Initial excretion rates were higher for intermolt (stage C) and late premolt (stage D_2) lobsters after the final hemolymph peak. Additionally, the excretion rate increased in mid-premolt (stage D_1), in the latter part of the 15-day observation period, at a time after injection equivalent to stage D_2 lobsters. The data indicate that excretion rates vary with the stage of the molt cycle; regulation of these excretion rates may therefore be an additional means of altering ecdysteroid titers. These results agree with earlier studies on insects. Hoffmann *et al.* (1974) and Koolman and Walter (1985) provided evidence that excretion rates varied and were lowest at times of peak hormone titer in locusts and blowflies. The role of excretion in regulating ecdysteroid titers in crustaceans remains obscure.

Excretion of [^{3}H]-ecdysteroids in lobster feces was detected following injection of [^{3}H]-ecdysone into hemolymph. These data confirm those from earlier RIA data (Snyder and Chang, 1991b), indicating that the lobster gut can absorb ecdysteroids from hemolymph and transform them into apolar conjugates prior to their excretion in feces. Apolar ecdysteroid conjugates were also found in larval crabs after the injection of [^{3}H]-ecdysone (Connat and Diehl, 1986). The apolar material in lobster feces consisted of conjugates of HP metabolites and 20,26E, 20E, ecdysone, and P. Apolar ecdysteroid-conjugating enzymes in the gut are, therefore, not specific for particular metabolites. Conjugating enzymes are similarly non-specific in spiders (Connat *et al.*, 1988c), ticks (Connat *et al.*, 1988b), mealworms (Delbecque *et al.*, 1988), and crickets (Whiting and Dinan, 1988). The apolar metabolites have been identified as long-chain fatty acid esters in a variety of arthropods, but their definitive identification in crustaceans awaits further study (Hoffman *et al.*, 1985; Kubo *et al.*, 1987; Whiting and Dinan, 1989). The failure of others to find apolar conjugates as major ecdysteroid metabolites of arthropods has been attributed to losses in purification or in the choice of HPLC conditions (Connat and Diehl, 1986).

When ingested by lobsters, [^{3}H]-ecdysone is converted to apolar conjugates without further metabolism to other ecdysteroids or absorption from the gut (Snyder and Chang, 1991b). Similarly, ingested ecdysteroids are efficiently "detoxified" to apolar metabolites in a variety of arthropods including spiders (Connat *et al.*, 1988c), ticks (Connat *et al.*, 1988a), and tobacco budworms (Kubo *et al.*, 1987). The role of the lobster midgut gland in apolar ecdysteroid conjugation was confirmed by injection of [^{3}H]-ecdysone (Table II; Fig. 7). Appreciable amounts of apolar conjugates were only found in the midgut gland. This finding parallels results derived from *in vitro* studies of the lobster (Snyder and Chang, 1992) and crayfish midgut glands (Gorell *et al.*, 1972). Appreciable amounts of apolar conjugates were also found in the crayfish midgut gland after injection of [^{3}H]-ecdysone into the hemolymph (Kuppert *et al.*, 1978). The function of the midgut gland is still unclear in relation to its role in ecdysteroid metabolism; it might be the slow release of apolar conjugates into the feces, or the provision, after hydrolysis, of an additional source of active hormone. Both Sehnal *et al.* (1981) and Williams (1987) have shown that the insect pupal midgut contains a mobilizable source of ecdysteroids that is sufficient to drive the pupal-adult transition

in the absence of prothoracic glands. The lack of appreciable absorption of [^{3}H]-ecdysone after ingestion by *H. americanus* argues that the sole function of this ecdysteroid metabolic route in crustaceans may be for excretion.

Apolar ecdysteroid conjugates have been found in ovaries and embryos in other arthropods, such as ticks (Connat *et al.*, 1988b), cockroaches (Slinger and Isaac, 1988), and crickets (Whiting and Dinan, 1988, 1989). Similar studies should be conducted on crustaceans to determine the presence of these apolar conjugates in ovaries and embryos.

In conclusion, the metabolism of [^{3}H]-ecdysteroids in *H. americanus* involves both polar and apolar pathways. The overall metabolic routes of lobster ecdysteroids are therefore similar to those found in a variety of other arthropods.

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Literature Cited

Aiken, D. E. 1973. Proecdysis, setal development, and molt prediction in the American lobster (*Homarus americanus*). *J. Fish. Res. Board Can.* **30:** 1337–1344.

Buchholz, F. 1982. The metabolism of ecdysone and its putative role as the female sex-pheromone in the green shore crab *Carcinus maenas* L. *Publ. Cent. Natl. Exploit. Oceans Actes Colloq.* **14:** 35–46.

Chang, E. S. 1989. Endocrine regulation of molting in Crustacea. *Rev. Aquatic Sci.* **1:** 131–157.

Chang, E. S., and D. E. Conklin. 1983. Lobster (*Homarus*) hatchery techniques. Pp. 271–275 in *CRC Handbook of Mariculture*, Vol. 1, J. P. McVey, ed. CRC Press, Boca Raton.

Chang, E. S., and J. D. O'Connor. 1977. Secretion of α-ecdysone by crab Y-organs *in vitro*. *Proc. Natl. Acad. Sci. USA* **74:** 615–618.

Chang, E. S., and J. D. O'Connor. 1978. *In vitro* secretion and hydroxylation of α-ecdysone as a function of the crustacean molt cycle. *Gen. Comp. Endocrinol.* **36:** 151–160.

Christ, B., and D. Sedlmeier. 1987. Variations in epidermal cyclic nucleotide-dependent protein kinase activity during moult cycle of the crayfish *Orconectes limosus* and hormonal control of kinase activity by 20-hydroxyecdysone. *Int. J. Biochem.* **19:** 79–84.

Conklin, D. E., and E. S. Chang. 1983. Grow-out techniques for the American lobster, *Homarus americanus*. Pp. 277–286 in *CRC Handbook of Mariculture*, Vol. 1, J. P. McVey, ed. CRC Press, Boca Raton.

Connat, J. L., and P. A. Diehl. 1986. Probable occurrence of ecdysteroid fatty acid esters in different classes of arthropods. *Insect Biochem.* **16:** 91–97.

Connat, J. L., E. M. Dotson, and P. A. Diehl. 1988a. Apolar conjugates of ecdysteroids are not used as a storage form of molting hormone in the argasid tick *Ornithodoros moubata*. *Arch. Insect Biochem. Physiol.* **9:** 221–235.

Connat, J. L., E. M. Dotson, and P. A. Diehl. 1988b. Metabolism of ecdysteroids in the female tick *Amblyomma hebraeum* (Ixodoidea, Ixodidae): accumulation of free ecdysone and 20-hydroxyecdysone in the eggs. *J. Comp. Physiol. B* **157:** 689–699.

Connat, J. L., P. A. Fürst, and M. Zweilin. 1988c. Detoxification of injected and ingested ecdysteroids in spiders. *Comp. Biochem. Physiol.* **91B:** 257–265.

Delbecque, J. P., J. L. Connat, and R. Lafont. 1988. Polar and apolar metabolites of ecdysteroids during the metamorphosis of *Tenebrio molitor*. *J. Insect Physiol.* **34:** 619–624.

Gilbert, L. I. 1989. The endocrine control of molting: the tobacco hornworm, *Manduca sexta*, as a model system. Pp. 448–471 in *Ecdysone: From Chemistry to Mode of Action*, J. Koolman, ed. Georg Thieme Verlag, Stuttgart.

Gorell, T. A., L. I. Gilbert, and J. B. Siddall. 1972. Binding proteins for an ecdysone metabolite in the crustacean hepatopancreas. *Proc. Natl. Acad. Sci. USA* **69:** 812–815.

Hampshire, F., and D. H. S. Horn. 1966. Structure of crustecdysone, a crustacean molting hormone. *Chem. Commun.* **2:** 37–38.

Hoffmann, J. A., J. Koolman, P. Karlson, and P. Joly. 1974. Molting hormone titer and metabolic fate of injected ecdysone during the fifth larval instar and in adults of *Locusta migratoria* (Orthoptera). *Gen. Comp. Endocrinol.* **22:** 90–97.

Hoffmann, K. H., D. Bulenda, E. Thiry, and E. Schmid. 1985. Apolar ecdysteroid esters in adult female crickets, *Gryllus bimaculatus*. *Life Sci.* **37:** 185–192.

Holliday, C. W. 1977. A new method for measuring the urinary rate of a brachyuran crab. *Comp. Biochem. Physiol.* **58A:** 119–120.

Hopkins, P. M. 1986. Ecdysteroid titers and Y-organ activity during late anecdysis and proecdysis in the fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol.* **63:** 362–373.

Isaac, R. E., and A. J. Slinger. 1989. Storage and excretion of ecdysteroids. Pp. 250–253 in *Ecdysone: From Chemistry to Mode of Action*, J. Koolman, ed. Georg Thieme Verlag, Stuttgart.

Koolman, J., and P. Karlson. 1985. Regulation of ecdysteroid titer: degradation. Pp. 343–361 in *Comprehensive Insect Physiology Biochemistry and Pharmacology*, Vol. 7, G. A. Kerkut and L. I. Gilbert, eds. Pergamon Press, Oxford.

Koolman, J., and J. Walter. 1985. Ecdysteroids in insects: how are their concentrations regulated? Pp. 198–220 in *Metamorphosis: British Society for Developmental Biology Symposium*, Vol. 8, M. Balls and M. Bownes, eds. Clarendon Press, Oxford.

Kubo, I., S. Komatsu, Y. Asaka, and G. De Boer. 1987. Isolation and identification of apolar metabolites of ingested 20-hydroxyecdysone in frass of *Heliothis virescens* larvae. *J. Chem. Ecol.* **13:** 785–794.

Kuppert, P., M. Büchler, and K.-D. Spindler. 1978. Distribution and transport of molting hormones in the crayfish, *Orconectes limosus*. *Z. Naturforsch.* **33C:** 437–441.

Lachaise, F., and R. Lafont. 1984. Ecdysteroid metabolism in a crab: *Carcinus maenas* L. *Steroids* **43:** 243–259.

Lachaise, F., M. Lagueux, R. Feyereisen, and J. A. Hoffmann. 1976. Métabolisme de l'ecdysone au cours du développement de *Carcinus maenas* (Brachyura, Decapoda). *C. R. Acad. Sci. Paris* **283:** 943–946.

Lachaise, F., M. Goudeau, C. Hetru, C. Kappler, and J. A. Hoffmann. 1981. Ecdysteroids and ovarian development in the shore crab, *Carcinus maenas*. *Hoppe-Seyler's Z. Physiol. Chem.* **363:** 1059–1067.

Lachaise, F., G. Carpenter, G. Sommé, J. Colandeau, and P. Beydon. 1989. Ecdysteroid synthesis by crab Y-organs. *J. Exp. Zool.* **252:** 283–292.

Lehmann, M., and J. Koolman. 1989. Regulation of ecdysone metabolism. Pp. 217–220 in *Ecdysone: From Chemistry to Mode of Action*, J. Koolman, ed. Georg Thieme Verlag, Stuttgart.

McCarthy, J. F. 1980. Ecdysone metabolism and the interruption of proecdysis in the land crab, *Gecarcinus lateralis*. *Biol. Bull.* **158:** 91–102.

McCarthy, J. F. 1982. Ecdysone metabolism in premolt land crabs (*Gecarcinus lateralis*). *Gen. Comp. Endocrinol.* **47:** 323–332.

Mitchell, M. J., and S. L. Smith. 1988. Ecdysone 20-monooxygenase activity throughout the life cycle of *Drosophila melanogaster*. *Gen. Comp. Endocrinol.* **72:** 467–470.

Mykles, D. L. 1980. The mechanism of fluid absorption at ecdysis in the American lobster, *Homarus americanus*. *J. Exp. Biol.* **84:** 89–101.

Sehnal, F., P. Maroy, and J. Mala. 1981. Regulation and significance of ecdysteroid titre fluctuations in lepidopterous larvae and pupae. *J. Insect Physiol.* **27:** 535–544.

Slinger, A. J., and R. E. Isaac. 1988. Synthesis of apolar ecdysone esters by ovaries of the cockroach *Periplaneta americana*. *Gen. Comp. Endocrinol.* **70:** 74–82.

Smith, S. L., W. E. Bollenbacher, and L. I. Gilbert. 1983. Ecdysone 20-monooxygenase activity during larval-pupal development of *Manduca sexta*. *Mol. Cell. Endocr.* **31:** 227–251.

Snyder, M. J., and E. S. Chang. 1991a. Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus*. I. Hemolymph titers and metabolites. *Gen. Comp. Endocrinol.* **81:** 133–145.

Snyder, M. J., and E. S. Chang. 1991b. Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus*. II. Excretion of metabolites. *Gen. Comp. Endocrinol.* (in press).

Snyder, M. J., and E. S. Chang. 1992. Role of the midgut gland in the metabolism and excretion of ecdysteroids by lobsters. *Homarus americanus*. *Gen. Comp. Endocrinol.* (in press).

Sokal, R. R., and F. J. Rohlf. 1969. *Biometry*. W. H. Freeman, San Francisco. 776 pp.

Soumoff, C., and D. M. Skinner. 1988. Ecdysone 20-monooxygenase activity in land crabs. *Comp. Biochem. Physiol.* **91C:** 139–144.

Spaziani, E., H. H. Rees, W. L. Wang, and R. D. Watson. 1989. Evidence that Y-organs of the crab *Cancer antennarius* secrete 3-dehydroecdysone. *Mol. Cell. Endocr.* **66:** 17–25.

Spindler, K.-D., A. Van Wormhoudt, D. Sellos, and M. Spindler-Barth. 1987. Ecdysteroid levels during embryogenesis in the shrimp, *Palaemon serratus* (Crustacea Decapoda): quantitative and qualitative changes. *Gen. Comp. Endocrinol.* **66:** 116–122.

Stevenson, J. R. 1968. Metecdysial molt staging and changes in the cuticle in the crayfish *Orconectes sanborni* (Faxon). *Crustaceana* **14:** 169–177.

Traub, M., G. Gellissen, and K.-D. Spindler. 1987. 20(OH)ecdysone-induced transition from intermolt to premolt protein biosynthesis patterns in the hypodermis of the crayfish, *Astacus leptodactylus*, *in vitro*. *Gen. Comp. Endocrinol.* **65:** 465–477.

Whiting, P., and L. Dinan. 1988. The occurrence of apolar ecdysteroid conjugates in newly-laid eggs of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **34:** 625–631.

Whiting, P., and L. Dinan. 1989. Identification of the endogenous apolar ecdysteroid conjugates present in newly-laid eggs of the house cricket (*Acheta domesticus*) as 22-long-chain fatty acyl esters of ecdysone. *Insect Biochem.* **19:** 759–765.

Williams, C. M. 1987. Midgut of lepidopteran pupae is a major depot of sequestered, mobilizable, ecdysteroids. *Memor. Instit. Oswaldo Cruz* **82:** 47–49.

Young, N. L. 1976. The metabolism of ^{3}H-molting hormone in *Calliphora erythrocephala* at the mature larval and white puparial stages. *Insect Biochem.* **6:** 1–12.

Young, N. L., and P. R. Young. 1976. Biochemical control of moulting hormone titer in *Calliphora erythrocephala* during puparium formation. *Insect Biochem.* **6:** 169–177.